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RESEARCH ARTICLE

Impaired mechanical response of an EDMD mutation leads to motility phenotypes that are repaired by loss of prenylation

Noam Zuela¹, Monika Zwerger², Tal Levin¹, Ohad Medalia^{2,3} and Yosef Gruenbaum^{1,*}

ABSTRACT

There are roughly 14 distinct heritable autosomal dominant diseases associated with mutations in lamins A/C, including Emery–Dreifuss muscular dystrophy (EDMD). The mechanical model proposes that the lamin mutations change the mechanical properties of muscle nuclei, leading to cell death and tissue deterioration. Here, we developed an experimental protocol that analyzes the effect of disease-linked lamin mutations on the response of nuclei to mechanical strain in living *Caenorhabditis elegans*. We found that the EDMD mutation L535P disrupts the nuclear mechanical response specifically in muscle nuclei. Inhibiting lamin prenylation rescued the mechanical response of the EDMD nuclei, reversed the muscle phenotypes and led to normal motility. The LINC complex and emerin were also required to regulate the mechanical response of *C. elegans* nuclei. This study provides evidence to support the mechanical model and offers a potential future therapeutic approach towards curing EDMD.

KEY WORDS: *C. elegans*, Emerin, Laminopathy, LINC complex, Mechanical strain, Nuclear envelope, Muscular dystrophy

INTRODUCTION

The nuclear lamina is a protein meshwork underlying the inner nuclear membrane (INM) that contains lamins and lamin-associated proteins. Lamins, the main components of the nuclear lamina, are evolutionary conserved nuclear intermediate filaments that are involved in most nuclear activities ranging from signal transduction and chromatin organization to DNA damage repair, replication and transcription (Burke and Stewart, 2014; Dechat et al., 2010; Gruenbaum and Foissner, 2015). Mammals have three lamin genes encoding four major isoforms termed lamins A, B1, B2 and C. Lamins A, B1 and B2 undergo farnesylation at their C-terminus. This modification causes the protein to become more hydrophobic, and is thought to facilitate the localization of lamin to the INM, as well as regulating protein–protein interactions. During the maturation process of lamins A, B1 and B2, the last 15 amino acids, including the farnesylated cysteine, are subsequently cleaved (Barrowman et al., 2008).

Tissue stiffness and stability is closely associated with a high lamin A to lamin B expression level ratio. Highly structured tissues show a high ratio of lamin A to lamin B, whereas soft tissues show a lower ratio (Swift et al., 2013). In addition, the ability of the nucleus to resist

stress and maintain its shape largely depends on lamin A; however, B-type lamins have also been shown to mediate nuclear shape and stiffness in neurons (Coffinier et al., 2011). Cells depleted of lamin A are less resistant to strain and become more deformable (Broers et al., 2004; Lammerding et al., 2006, 2004), whereas a higher expression of lamin A and knockdown of lamin B1 stiffens nuclei and impairs cellular migratory capabilities (Harada et al., 2014; Rowat et al., 2013; Shin et al., 2013). One of the defining domains in lamin A that determines its visco-elastic properties is the Ig fold (Bera et al., 2014). When lamin A is exposed to shear stress in isolated nuclei, the Ig fold partially unfolds leading to stretching of the lamin molecule (Swift et al., 2013). Increasing extracellular matrix stiffness coupled to myosin-II activity causes dephosphorylation of S22 residue on lamin A. The dephosphorylation of S22 causes nucleoplasmic lamin A to localize to the nuclear lamina, which provides increased nuclear stiffness and mechanical support (Buxboim et al., 2014; Guilluy and Burridge, 2015). This shift in lamin A distribution can also affect signal transduction and chromatin organization (Isermann and Lammerding, 2013). The LINC complex connects nuclear lamina, cytoplasmic organelles and filaments and is therefore involved in determining the scaffolding properties of the nucleus (Crisp and Burke, 2008; Lee et al., 2002; Starr and Fischer, 2005; Tzur et al., 2006). In summary, nuclei can sense external forces and can modulate both the lamina and the nuclear envelope composition accordingly.

There are at least 14 distinct diseases caused by over 400 different missense mutations in the *LMNA* gene, which encodes lamins A and C. Most of these diseases, collectively termed laminopathies, are autosomal dominant and appear postnatally. Laminopathies are roughly divided into four major disease groups: muscle diseases, lipodystrophies, peripheral neuropathies and accelerated aging disorders (Gruenbaum and Foissner, 2015; Schreiber and Kennedy, 2013; Worman, 2012). In the case of essentially all *LMNA* mutations affecting load-bearing tissues such as striated muscles, smooth muscles, cartilages and bone, the nucleo-skeletal coupling is impaired. Cells deficient in lamin A, or expressing certain lamin disease mutations display changes in nuclear stability and deformability (Folker et al., 2011; Zwerger et al., 2013). One of the models attempting to explain laminopathies is the mechanical model. According to this model, a defective nuclear mechanical response originating from specific lamin A mutations causes cells to be more susceptible to damage, leading to cell death and progressive tissue deterioration in mechanically stressed tissues (Zwerger et al., 2011). To date, the changes in nuclear deformation, in response to strain application, caused by lamin mutations have been demonstrated only on isolated cells or tissue (Zwerger et al., 2013). It is still uncertain, however, how globally expressed mutations cause tissue-specific effects.

Caenorhabditis elegans have one constitutively expressed lamin protein (encoded by *lmn-1*) which is homologous to the human lamin B and is permanently farnesylated. Despite this, *lmn-1* shares many attributes and functions with lamin A, including a presence in

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the nucleoplasm, interactions with binding partners that in mammals interact with lamin A, as well as a role in regulating the mechanical properties of the nucleus (Liu et al., 2000; Lyakhovetsky and Gruenbaum, 2014). Many of the lamin residues that cause diseases (Wiesel et al., 2008) are conserved in the *C. elegans* lamin. These dominantly expressed disease-linked mutations cause muscle or aging phenotypes that resemble the phenotypes in human patients (Bank and Gruenbaum, 2011). We set out to study the contribution of changes in nuclear mechanical response to better understand disease progression by analyzing the effects of an Emery–Dreifuss muscular dystrophy (EDMD)-linked lamin mutation on nuclear mechanical response in the context of a living organism. We also show that improving the aberrant nuclear mechanical response caused by this EDMD mutation has beneficial effects on animal movement and muscle organization.

RESULTS

Nuclear response to mechanical strain in living *C. elegans*

We have adapted an established nuclear strain application system, which measures the response of nuclei to external strain application (Lammerding and Lee, 2009), in order to measure the response of *C. elegans* nuclei from different tissues to mechanical strain *in vivo*.

In this system, animals expressing either wild-type lamin::GFP or lamin carrying disease-linked mutations tagged with GFP are glued to a silicone membrane with surgical glue at their head and tail regions. The membrane is stretched to ~140% of its original length (Fig. 1A, dark red arrows). The length of the nuclei is then measured using a fluorescence microscope along the head to tail axis, at the plane of their maximal length before, during and after strain application. The change in nuclear length due to the strain application is calculated and normalized to the change in length between neighboring nuclei (normalized nuclear strain; Fig. 1A). In addition, we analyzed the ability of nuclei to return to their original length (residual strain) (Fig. 1B).

Tissue-specific response to mechanical strain of nuclei expressing an EDMD-linked mutation

Our previous studies have shown that, similar to human lamin A, the single *C. elegans* lamin protein is required for maintaining the shape of nuclei (Liu et al., 2000). Previously, we have generated *C. elegans* strains expressing lamin containing missense homologous mutations to those that lead to laminopathic diseases in humans (Table 1; Bank et al., 2011; Wiesel et al., 2008). Despite the fact that the expression level of the mutant lamin::GFP constructs is

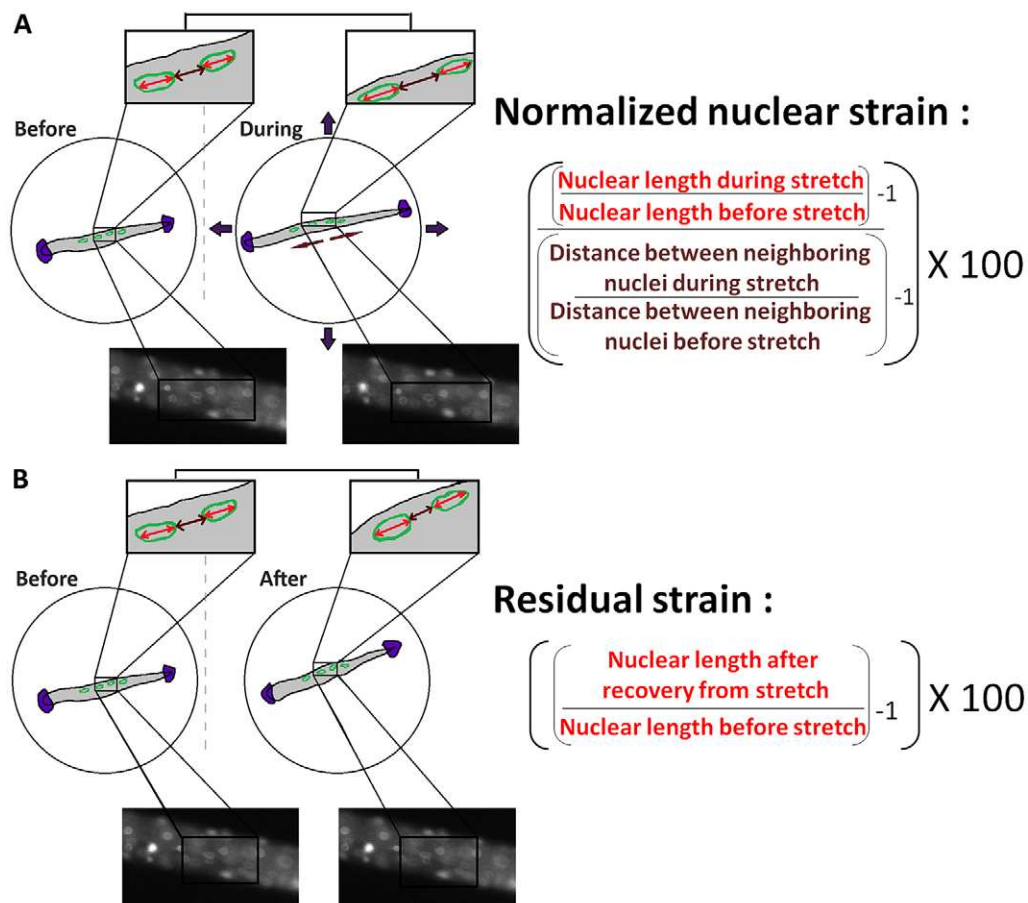


Fig. 1. Experimental concept of measuring nuclear mechanical responses to external strain application. A late larval L4 animal is glued at both ends to a deformable silicone membrane. The membrane is placed on the stretching device under a fluorescence microscope. Images of GFP::lamin are taken before stretching, at maximal stretching and after relaxation from a 1 min stretch. (A) Analysis of nuclear mechanical response to external strain application in intact *C. elegans*. Normalized nuclear strain was measured as the longitudinal deformation of the nucleus during external strain application (bright red arrow, right panel) along the stretch axis (directionality of bright and dark red arrows) compared to the initial length of the same nucleus (bright red arrow, left panel) before strain application, normalized to the longitudinal deformation of the surrounding tissue (dark red arrows) along the stretch axis. (B) Analysis of nuclear recovery from mechanical strain. Residual strain was measured as the length of the nucleus (light red arrow, left panel) along the stretch axis (directionality of bright and dark red arrows) before the application of external strain (light red arrow, left panel) compared to its length along the stretch axis after the strain had been eliminated (bright red arrow, right panel).

Table 1. Disease-linked LMNA mutations used in this study

Human mutation	<i>C. elegans</i> mutation	Disease	Ref.
L215P	R460C	No disease	
	L229P	Dilated cardiomyopathy (DCM)	Cowan et al. (2010)
G465D	G472D	Familial partial lipodystrophy (FPLD)	Speckman et al. (2000)
E145K	Q159K	Hutchinson–Gilford progeria syndrome (HGPS)	Taimen et al. (2009)
L530P	L535P	Emery–Dreifuss muscular dystrophy (EDMD)	Bonne et al. (1999)

For details, see references Bonne et al., 1999, Cowan et al., 2010, Speckman et al., 2000 and Taimen et al., 2009.

only ~15% of the endogenous lamin background, it leads to muscle and aging phenotypes that closely resemble the human diseases (Bank et al., 2011; Bank and Gruenbaum, 2011; Wiesel et al., 2008).

Here, we tested the normalized nuclear strain of nuclei in muscle, hypodermis and neuronal tissues. Animals expressing wild-type lamin::GFP were compared with animals expressing emerin::GFP that were transfected with an empty vector or animals expressing the R460C lamin mutation. Normalized nuclear strain values were similar in all of these animals (28±5%; mean±s.e.m.) (Fig. 2A,B). Similarly, applying external strain application to animals expressing the lamin::GFP mutations L229P (dilated cardiomyopathy, DCM) or G472D (familial partial lipodystrophy, FPLD) (Table 1) showed

non-significant changes compared to animals expressing wild-type lamin::GFP (Fig. 2A,B). In both muscle and hypodermis cells, *lmn-1* depletion [by RNA interference (RNAi)] increased nuclei deformability when subjected to external strain application (normalized nuclear strain was 66% in muscle nuclei and normalized nuclear strain was 60% in hypodermal nuclei) (Fig. 2A,B). In contrast, in both tissues, the Q159K progeria-linked mutation caused increased nuclear rigidity in response to external strain application (normalized nuclear strain of 13% in both tissues). The L535P EDMD-linked lamin mutation in the Ig fold domain significantly increased the resistance of muscle nuclei to external strain application (normalized nuclear strain of 14%; Figs 2A and 3A), whereas in hypodermal nuclei expressing the L535P lamin mutation, the response to mechanical strain was similar to that of nuclei expressing wild-type lamin::GFP (Figs 2B and 3A). Not all EDMD lamin mutations showed the same change in mechanical response seen in L535P. For example, the Y59C EDMD-linked lamin mutation in the rod domain had no significant effect on the mechanical response of either muscle or hypodermal nuclei to external strain application (normalized nuclear strain was 25% in muscle nuclei and 26% in hypodermal nuclei) (Fig. 2A,B). To the best of our knowledge, this is the first indication for a tissue-specific response to mechanical strain of nuclei in animals globally expressing a muscle laminopathic mutation.

We then tested the ability of nuclei expressing laminopathic-linked mutations to recover from the mechanical strain. The residual strains of muscle and hypodermis nuclei expressing wild-type lamin::GFP were not significant (Fig. 2C,D). Non-significant changes were also observed in animals expressing lamin R460C (Fig. 2C,D) and in

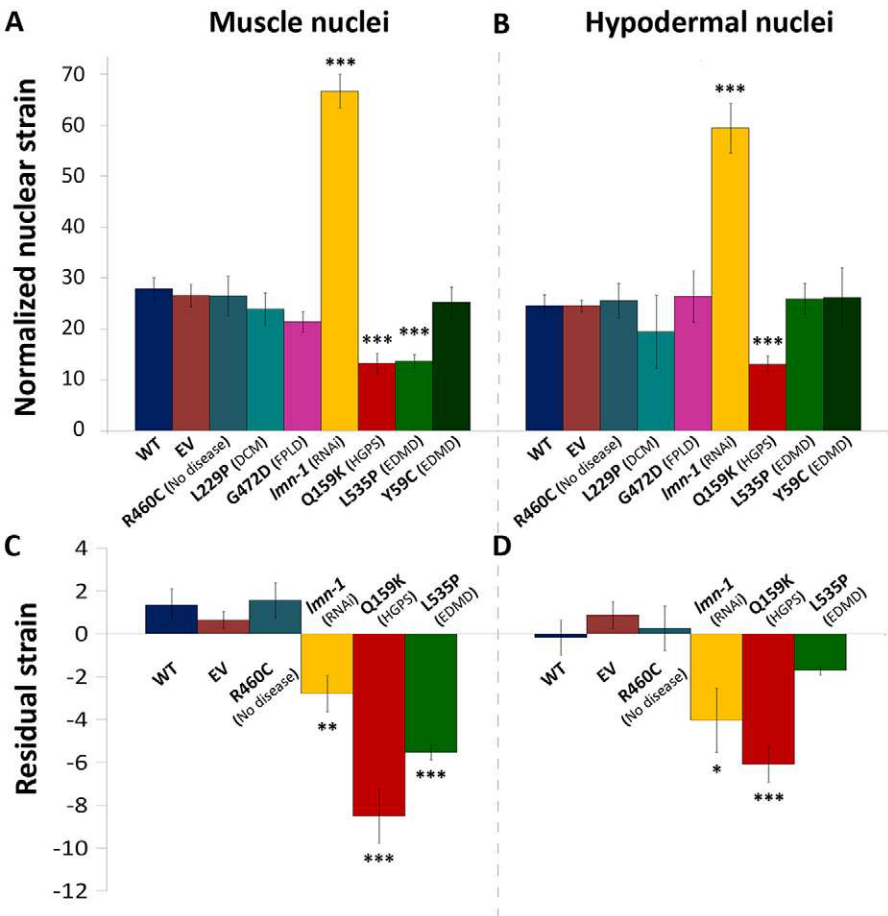


Fig. 2. The EDMD lamin mutation L535P causes alterations in the response of muscle nuclei to mechanical strain. The mechanical response in different living *C. elegans* strains to external strain application was measured in muscle (A) and hypodermis (B) nuclei. Emerin::GFP was used when *lmn-1* was downregulated. WT, wild-type lamin::GFP; EV, a strain expressing emerin::GFP that was subjected to feeding with an empty vector. The *P*-values for the different normalized nuclear strain experiments were 0.67 ($n=27$), 2×10^{-13} ($n=31$), 0.76 ($n=12$), 0.42 ($n=45$), 0.78 ($n=12$), 6.34×10^{-6} ($n=22$), 7.08×10^{-7} ($n=34$) and 0.96 ($n=28$) in A, and 0.98 ($n=19$), 0.0002 ($n=18$), 0.41 ($n=8$), 0.08 ($n=17$), 0.72 ($n=3$), 0.002 ($n=12$), 0.74 ($n=16$) and 0.16 ($n=8$) in B for EV, *lmn-1* (RNAi), R460C, G472D, L229P, Q159K, L535P and Y59C, respectively. The residual strain in different living *C. elegans* strains after relaxation from external strain application was measured in muscle (C) and hypodermis (D) nuclei. The *P*-values for the different residual strain were: 0.41 ($n=19$), 0.86 ($n=33$), 0.0006 ($n=25$), 2.5×10^{-7} ($n=18$) and 1.59×10^{-10} ($n=38$) in (C) and 0.31 ($n=17$), 0.75 ($n=25$), 0.03 ($n=26$), 9.44×10^{-6} ($n=21$) and 0.08 ($n=21$) in (D) for EV, R460C, *lmn-1* RNAi, Q159K and L535P, respectively. *P*-values are compared to wild-type lamin::GFP and were calculated by performing a *t*-test. Results represent mean±s.e.m. **P*<0.05, ***P*<0.005, ****P*<0.0005.

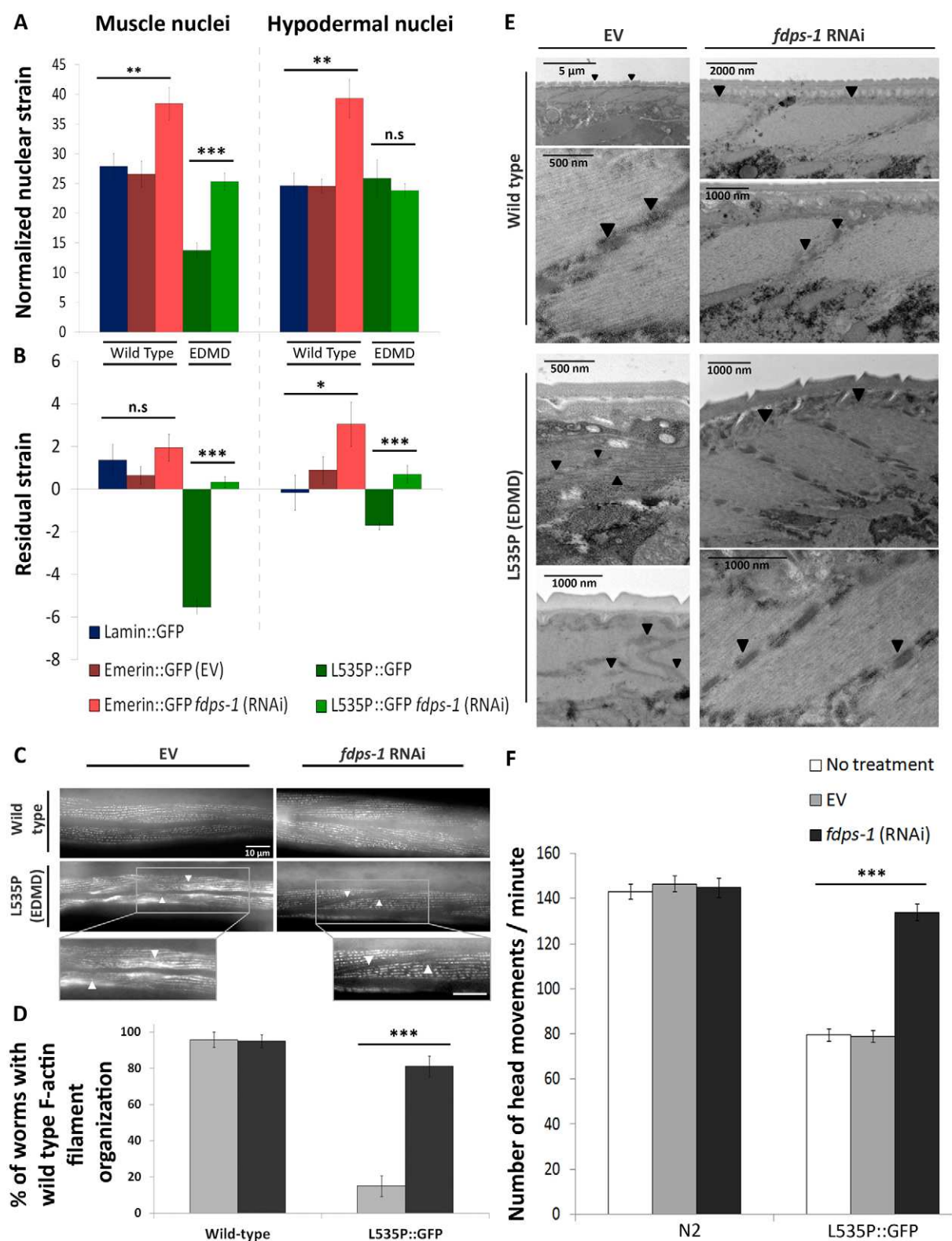


Fig. 3. See next page for legend.

those treated with an empty vector. *lmn-1* (RNAi) impaired the ability of nuclei to return to their original length by causing an inward collapse of the nuclei (residual strain was -3% and -4% in muscle and hypodermis nuclei, respectively). The Q159K progeria-linked lamin mutation also caused an inward collapse of nuclei in both

tissues (residual strain was -9% and -5% in muscle and hypodermis nuclei, respectively). Again, the L535P EDMD-linked lamin mutation showed a muscle-specific effect with residual strain of -5.5% in muscle nuclei, whereas the change in hypodermal nuclei (residual strain of -1%) was not significant (Fig. 2B).

Fig. 3. Downregulation of *fdps-1* rescues both the nuclear mechanical response and disease phenotypes of L535P animals. (A) Normalized nuclear strain values were measured in response to *fdps-1* (RNAi) treatment in muscle (left panels) and hypodermis (right panels) nuclei. Significant changes were observed in *fdps-1* (RNAi) of wild-type nuclei in both muscle and hypodermis compared to untreated nuclei [$P=0.004$ ($n=24$) and 0.001 ($n=13$), respectively]. A significant change was also observed in L535P muscle nuclei subjected to *fdps-1* (RNAi) ($P=8.2\times 10^{-8}$; $n=34$) compared to untreated L535P nuclei. (B) Recovery from mechanical strain was analyzed in muscle (left panel) and hypodermis (right panel) nuclei. Significant changes were observed in *fdps-1* (RNAi) of L535P muscle nuclei ($P=1.2\times 10^{-19}$; $n=24$) and hypodermis nuclei ($P=6.2\times 10^{-6}$; $n=30$). Results represent mean \pm s.e.m. (C) Phalloidin staining in *C. elegans* subjected to empty vector (EV) (left panels) or *fdps-1* (RNAi) (right panels). Top panels, wild-type lamin; middle panels, animals expressing the L535P lamin mutation; bottom panels, enlargement of a region taken from the middle panels. Arrowheads, actin filaments. Scale bar: 10 μ m. (D) Quantification of actin filament organization following phalloidin staining. *fdps-1* (RNAi) treatment of L535P animals ($n=48$) rescued filament organization as compared to empty-vector-treated L535P animals ($n=40$; $P=2.1\times 10^{-12}$). (E) Electron microscopy analysis of the effects of *fdps-1* (RNAi) on muscle organization in *C. elegans* subjected to treatment with empty vector (left panels) or *fdps-1* (RNAi) (right panels). In animals expressing wild-type lamin (top panels), *fdps-1* (RNAi) treatment did not cause a change in muscle organization (black arrowheads). In contrast, *fdps-1* (RNAi) rescued the aberrant muscle morphology observed in animals expressing the L535P mutation (black arrowheads in bottom panels). (F) Analysis of animal motility ($n=20$ for each treatment) in wild-type and L535P strains in response to *fdps-1* (RNAi) treatment. A significant change was observed in the L535P animals following *fdps-1* RNAi treatment ($P=2.4\times 10^{-13}$). P -values were calculated by performing a t -test. Results represent mean \pm s.e.m. * $P<0.05$, ** $P<0.005$, *** $P<0.0005$; n.s., not significant.

With the exception of lamin mutations that cause Charcot–Marie–Tooth disease (Worman, 2012), lamin mutations do not affect neuronal cells. Indeed, none of the tested lamin mutations affected the response of *C. elegans* neuronal nuclei to mechanical strain. Changes in both normalized nuclear strain and residual strain values were not significant in all tested strains (Fig. S1).

One possible explanation for the changes in the response of nuclei to mechanical strain could be differences in expression levels of mutant lamins in the nuclei of different tissues. However, there was no direct correlation between the expression levels of L535P mutant lamin, after normalization to the nuclear area, and the tissue-specific mechanical response (Fig. S2A). Another explanation might be differential localization of the mutant lamin favoring the nuclear periphery in Q159K nuclei and L535P muscle nuclei. Analyzing lamin distribution with respect to the nuclear periphery showed that the nuclear periphery in Q159K-expressing nuclei was not significantly enriched for lamin compared to nuclear periphery in wild-type nuclei. In L535P-expressing muscle nuclei there was no significant peripheral enrichment as compared to hypodermal and neuronal nuclei (Fig. S2B). It is apparent that animals expressing the EDMD mutation L535P or the progeroid mutation Q159K showed decreased nuclear deformation and reduced ability to re-establish their initial length when subjected to strain. Notably this effect is tissue specific in the case of EDMD but not in the case of Hutchinson–Gilford progeria syndrome (HGPS), and is not correlated to lamin expression levels or its nuclear distribution.

Loss of prenylation restores the mechanical response of muscle nuclei in L535P EDMD animals

The farnesyl diphosphate synthase gene (*fdps-1*) is required for both farnesylation and geranyl-geranylation. In *C. elegans*, depletion of *fdps-1* (using RNAi) in wild-type (N2) animals rejuvenates the shape of aging nuclei, but does not affect aging or motility (Bar and Gruenbaum, 2010). However, subjecting animals

expressing wild-type lamin::GFP or the L535P mutant lamin::GFP to *fdps-1* (RNAi) caused a small shift in lamin distribution from the nuclear periphery to the nucleoplasm in muscle cells (6% and 9%, respectively) (Fig. S3B).

The *fdps-1* (RNAi) treatment of wild-type animals expressing *emerin::GFP* caused both muscle and hypodermal nuclei to show less resistance to mechanical strain (normalized nuclear strain of 38% and 39%, respectively) (Fig. 3A). However, there was no significant effect on their ability to recover from mechanical strain (Fig. 3B). In contrast, there was a reversion of the muscle nuclear response to external strain application upon *fdps-1* (RNAi) treatment of animals expressing the L535P lamin mutation, making their normalized nuclear strain value similar to that of animals expressing wild-type lamin (Fig. 3A). The recovery of the L535P muscle nuclei from the mechanical strain was also similar to that of animals expressing wild-type lamin (Fig. 3B). There was no effect of external strain application on the normalized nuclear strain or residual strain values of hypodermal nuclei expressing the L535P lamin mutation and subjected to *fdps-1* (RNAi) (Fig. 3A,B). The effect was specific to nuclei expressing the L535P lamin mutation, because *fdps-1* (RNAi) neither affected the nuclear mechanical response of the progeria-linked Q159K mutation (Fig. S4A,B) nor the nuclear distribution of the Q159K lamin mutation (Fig. S3B). The small shift in mutant lamin from the nuclear periphery to the nucleoplasm caused by *fdps-1* (RNAi) only had beneficial effects on the mechanical response of muscle nuclei in animals expressing the L535P lamin mutation.

Loss of prenylation rescues muscle structure and motility in L535P EDMD animals

We next analyzed whether the rescue of normalized nuclear strain and residual strain in nuclei expressing the L535P lamin mutation and subjected to *fdps-1* (RNAi) also affects muscle morphology and animal motility. Phalloidin staining of *fdps-1* (RNAi) L535P-expressing late L4 animals showed rescue of actin filament organization as compared to animals expressing the wild-type lamin. Approximately 85% of L535P-expressing animals treated with *fdps-1* (RNAi) demonstrated wild-type actin filament organization, as compared to 95% in wild-type animals and only 15% in L535P animals treated with an empty vector (Fig. 3C,D; Bank et al., 2012). The rescue of the muscle phenotype was further analyzed in late L4 animals by thin-section transmission electron microscopy (Fig. 3E). Although muscle morphology was altered with abnormal filament organization and loss of the uniform size and shape of sarcomeres in animals expressing the L535P mutant lamin, the same animals subjected to *fdps-1* (RNAi) showed normal muscle morphology (Fig. 3E).

Previous studies have shown that *fdps-1* (RNAi) does not affect animal motility and muscle organization in animals expressing wild-type lamin (Bar and Gruenbaum, 2010) (Fig. 3C–E). In contrast, animals expressing the L535P lamin mutation, in addition to their muscle phenotypes, show reduced motility (Bank et al., 2012) (Fig. 3F). In light of the rescue of the nuclear mechanical response and muscle phenotypes in L535P-expressing animals, we analyzed whether the same treatment affects animal motility. Downregulation of *fdps-1* in animals expressing the EDMD-linked L535P mutation led to complete rescue of their motility (Fig. 3F). In animals expressing the progeria-linked Q159K mutation, both muscle organization and motility remained impaired following *fdps-1* (RNAi) (Fig. S4). Given that the rescue in muscle structure and motility was specific to the L535P mutation, we assume that restoring mechanical response of nuclei can improve tissue structure and function.

Disruption of the LINC complex increases nuclear stiffness in L535P EDMD animals

The ‘linker of nucleoskeleton and cytoskeleton’ (LINC) complex is composed of SUN-domain- and KASH-domain-containing proteins (Starr, 2009; Tzur et al., 2006). It bridges across the inner and outer nuclear membranes and physically connects the nucleus to every major component of the cytoskeleton (Crisp et al., 2006). *C. elegans* have two SUN-domain proteins: UNC-84 and matefin (also known as SUN-1). Whereas UNC-84 is expressed throughout development, matefin is only present in the germline and early embryos (Fridkin et al., 2004; Lee et al., 2002). In human EDMD patients, SUN1 and SUN2 can act as disease modifier genes, aggravating disease phenotypes (Meinke et al., 2014). These EDMD patient cells show defective nuclear force transmission and impaired nuclear stability (Folker et al., 2011; Zwerger et al., 2013). Therefore, we analyzed the effect of the LINC complex on the response of nuclei to external strain application. Even though UNC-84 requires lamin for its proper nuclear envelope localization, UNC-84 does not affect lamin nuclear localization (Lee et al., 2002). Indeed, subjecting animals expressing wild-type lamin::GFP or L535P mutant lamin tagged to GFP to *unc-84* depletion (RNAi) led to no effect on the nuclear distribution of lamin in either muscle or hypodermal nuclei (Fig. 4D). Downregulation of *unc-84* had no significant effect on muscle mechanical response of animals expressing emerin::GFP. The change in normalized nuclear strain from 25% to 40% and residual strain from -0.1% to 4% in hypodermal cells, however, was statistically significant (Fig. 4A,B), suggesting that there is a role for the LINC complex in determining the response of *C. elegans* hypodermal nuclei to external strain application. By contrast, there was no effect of *unc-84* (RNAi) on the normalized nuclear strain of hypodermal nuclei expressing the L535P lamin mutation, but a significant change was seen in residual strain from -5.5% to 3% , which is still comparable to that of wild-type. However, the normalized nuclear strain of L535P muscle nuclei subjected to *unc-84* (RNAi) changed significantly from that of untreated L535P muscle nuclei (normalized nuclear strain was 9% versus 14%) and upon release of strain these nuclei were able to better recover (residual strain was 2% vs 5% ; Fig. 4B).

Downregulation of *unc-84* in L4 wild-type *C. elegans* had no effect on animal mobility (Fig. 4C). Interestingly, although *unc-84* (RNAi) partially restored the ability of stretched L535P muscle nuclei to return to their original length, the increase in nuclear stiffness was correlated with a significant worsening of the motility phenotype (Fig. 4C). These results suggest that the LINC complex, in combination with the L535P mutation, affects both muscle nuclear mechanics and animal motility, and indicates a close association between nuclear stiffness and animal motility.

Emerin regulates nuclear mechanics in both muscle and hypodermis

Another inner nuclear membrane protein involved in the response of nuclei to external strain application is emerin (Ho et al., 2013; Lammerding et al., 2005). Downregulation of *emr-1* had no significant effect on normalized nuclear strain in both muscle and hypodermis nuclei expressing wild-type lamin::GFP (Fig. 5A). However, the nuclei of both tissues remained bigger after removal of the strain application (residual strain was 4% for both tissues; Fig. 5B). Downregulating *emr-1* in animals expressing the L535P lamin mutation did not significantly affect the response of muscle nuclei to external strain application (Fig. 5A). Interestingly, *emr-1* (RNAi) caused increased nuclear rigidity in hypodermal nuclei (normalized nuclear strain was 9% ; Fig. 5A).

Although *emr-1* (RNAi) slightly improved the ability of hypodermal nuclei expressing L535P lamin mutation to return to their original shape, it rescued the inward collapse phenotype in muscle nuclei (residual strain was 2% versus -5% ; Fig. 5B). We therefore analyzed the effects of *emr-1* (RNAi) on the motility of wild-type animals or animals expressing the L535P lamin mutation. In both strains *emr-1* (RNAi) had no effect on animal mobility (Fig. 5C). We conclude that emerin is involved in mediating nuclear recovery from mechanical strain, and in combination with the L535P EDMD-linked lamin mutation, effects hypodermal nuclear deformability.

DISCUSSION

In this study, we analyzed the effects of several disease-causing mutations in lamin on the nuclear mechanical response to external strain application in various tissue types within a living organism. Our experimental system allows the study of the dominant effects of evolutionary conserved laminopathy-causing mutations, as well as taking into account the different forces working in and around the tissue, the progressive nature of these diseases and the involvement of different genes in mediating this response.

One of the prominent models trying to explain muscle laminopathies such as EDMD is the mechanical model (Gruenbaum et al., 2005; Hutchison and Worman, 2004). This model proposes that certain lamin mutations disrupt the proper mechanical response of nuclei. Given that nuclei of load-bearing tissues are subject to intense mechanical forces, the impaired response is more apparent in them, leading to cellular death and tissue deterioration. Indeed, the L535P EDMD-linked lamin mutation, which is located at the Ig fold region of the lamin tail domain, causes increased nuclear rigidity and reduced deformation capabilities in nuclei of muscle cells, but not in other tested nuclei. Given that the Ig fold is a major contributor to the viscoelastic properties of lamin during strain application (Swift et al., 2013), it is conceivable that the L535P mutation causes a decrease in lamin elasticity, which in turn increases nuclear resistance to strain in a dominant way. An alternative reason for the reduced deformability of L535P muscle nuclei might be disruption of the force transmission process from the cytoskeleton to the nucleus by the L535P lamin mutation. In this case, the L535P muscle nuclei would sense less strain and consequently deform to a lesser extent than wild-type nuclei. Given that we see a dominant effect on the mechanical response even when the expression of the mutant lamin is relatively low ($\sim 15\%$ of wild-type expression), a higher mutant lamin expression could lead to a more severe phenotype. It is important to note that the rod domain Y59C EDMD-linked lamin mutation had no effect on both muscle and hypodermis nuclei deformability. This suggests that even though most EDMD lamin mutations disrupt nucleo-skeletal coupling, not all EDMD mutations affect the nuclear mechanical response. Ideally, experiments should be complemented by independent approaches to directly measure nuclear deformability in different cell types, for example, by atomic force microscopy (AFM) or micropipette aspiration. To date, however, there are no experimental procedures in living *C. elegans* that allow performing these types of analyses. One of the reasons is the lack of tissue cultures, as well as the lack of cell isolation procedures for adult *C. elegans*.

Previous studies have reported that *Lmna*^{-/-} mouse adult fibroblasts (MAFs) have impaired cytoplasmic mechanical properties (Hale et al., 2008). In contrast, expression of lamin A containing the L530P human mutation, which corresponds to the *C. elegans* L535P mutation, did not change nuclear

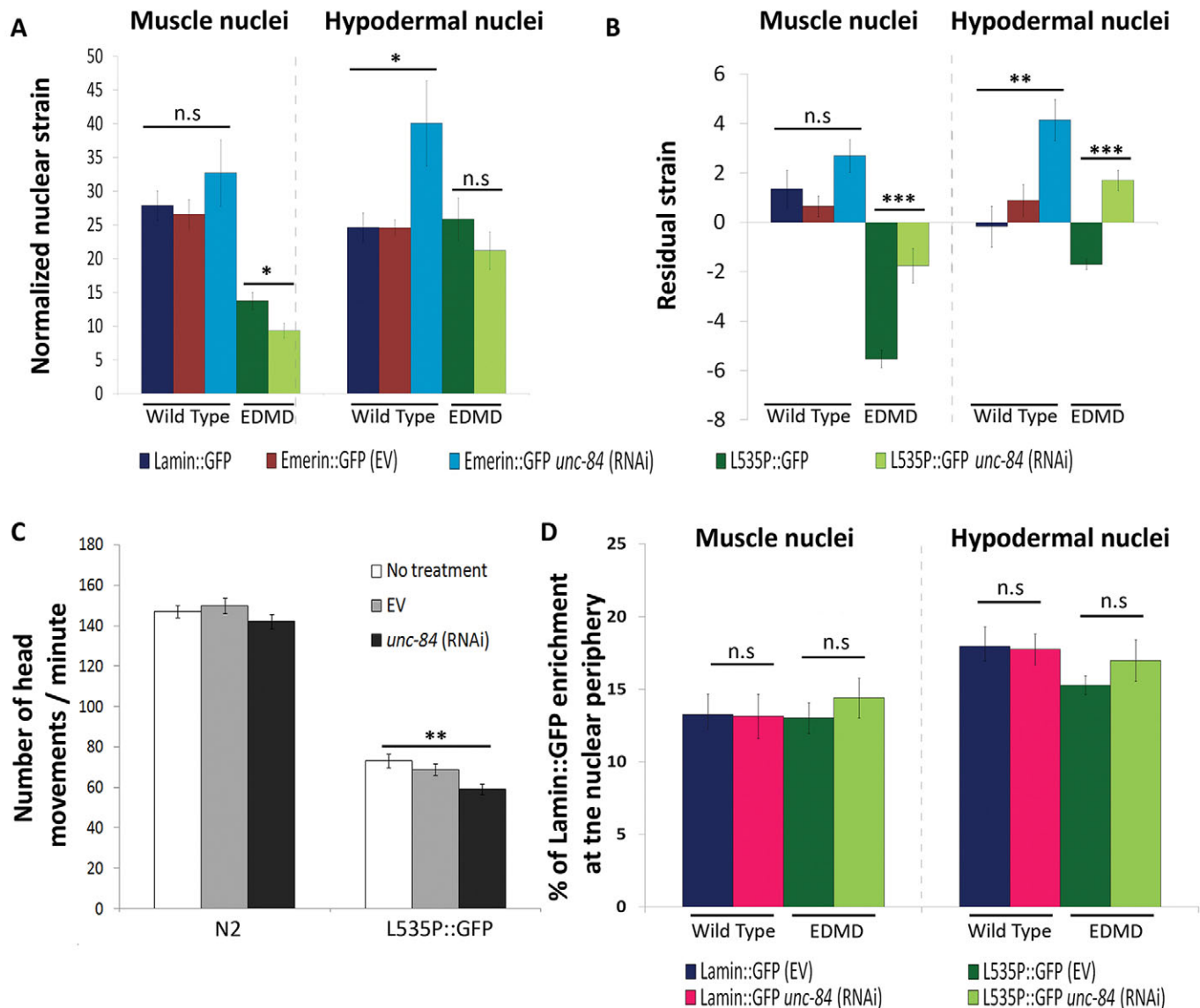


Fig. 4. Downregulation of *unc-84* affects both the nuclear mechanical response and motility of L535P animals. (A) Normalized nuclear strain values were measured in response to *unc-84* (RNAi) treatment in muscle (left panel) and hypodermis (right panel) nuclei. Significant changes were observed in L535P muscle nuclei ($P=0.01$; $n=18$ compared to untreated L535P nuclei) and in *unc-84* (RNAi) wild-type hypodermal nuclei ($P=0.03$; $n=15$ compared to untreated wild-type hypodermal nuclei). (B) Recovery from mechanical strain was analyzed in muscle (left panel) and hypodermis (right panel) nuclei. Significant changes were observed in L535P *unc-84* (RNAi) muscle nuclei compared to untreated L535P ($P=0.0002$; $n=11$), in wild-type hypodermal nuclei subjected to *unc-84* (RNAi) compared to wild-type nuclei ($P=0.0007$; $n=17$) and in hypodermal L535P nuclei subjected to *unc-84* (RNAi) ($P=8.8 \times 10^{-7}$; $n=13$ compared to untreated hypodermal L535P nuclei). (C) Analysis of animal motility in wild-type ($n=25$) and L535P ($n=30$) strains in response to *unc-84* (RNAi) treatment. A significant change was observed in the L535P animals following *unc-84* RNAi treatment ($P=0.001$). (D) Analysis of lamin::GFP distribution in nuclei of animals downregulated for *unc-84*. Shown is the enrichment (peripheral to nucleoplasmic ratio) of lamin::GFP (wild-type or mutant) in the nuclear periphery. No significant changes in lamin distribution were observed in either wild-type animal muscle [empty vector (EV), $n=20$, *unc-84* (RNAi), $n=28$] and hypodermal [empty vector, $n=29$, *unc-84* (RNAi), $n=33$] nuclei, or in L535P animal muscle [empty vector, $n=26$; *unc-84* (RNAi), $n=28$] and hypodermal [empty vector, $n=39$; *unc-84* (RNAi), $n=23$] nuclei. P -values were calculated by performing a t -test. Results represent mean \pm s.e.m. * $P<0.05$, ** $P<0.005$, *** $P<0.0005$; n.s., not significant.

deformability of *Lmna*^{+/-} mouse embryonic fibroblasts (MEFs), and in *D. melanogaster* body-wall muscle filets, the expression of the corresponding mutation caused nuclei to become slightly more deformable (Zwerger et al., 2013). There are several potential explanations for this difference. First, that unlike *C. elegans* and human, where lamin mutations lead to disease phenotypes in a dominant manner, mice heterozygous for many laminopathic EDMD mutations do not show signs of disease and only show muscle phenotypes in the homozygous state. Second, that the progressive nature of many laminopathies causes phenotypes to

appear only in the adult stage. For example, the progeria MEFs do not really show any of the aging phenotypes seen in MAFs (Hernandez et al., 2010). Studying nuclear mechanical response at the appropriate developmental stage facilitates the detection of phenotypes, which could otherwise be undetectable. To the best of our knowledge, this is the first report of tissue-specific mechanical response to a ubiquitously expressed lamin mutation, which is in agreement with the mechanical model. There are two main possible explanations for the tissue-specific response of ubiquitously expressed mutant lamin: (1) that the mechanical strain on muscle

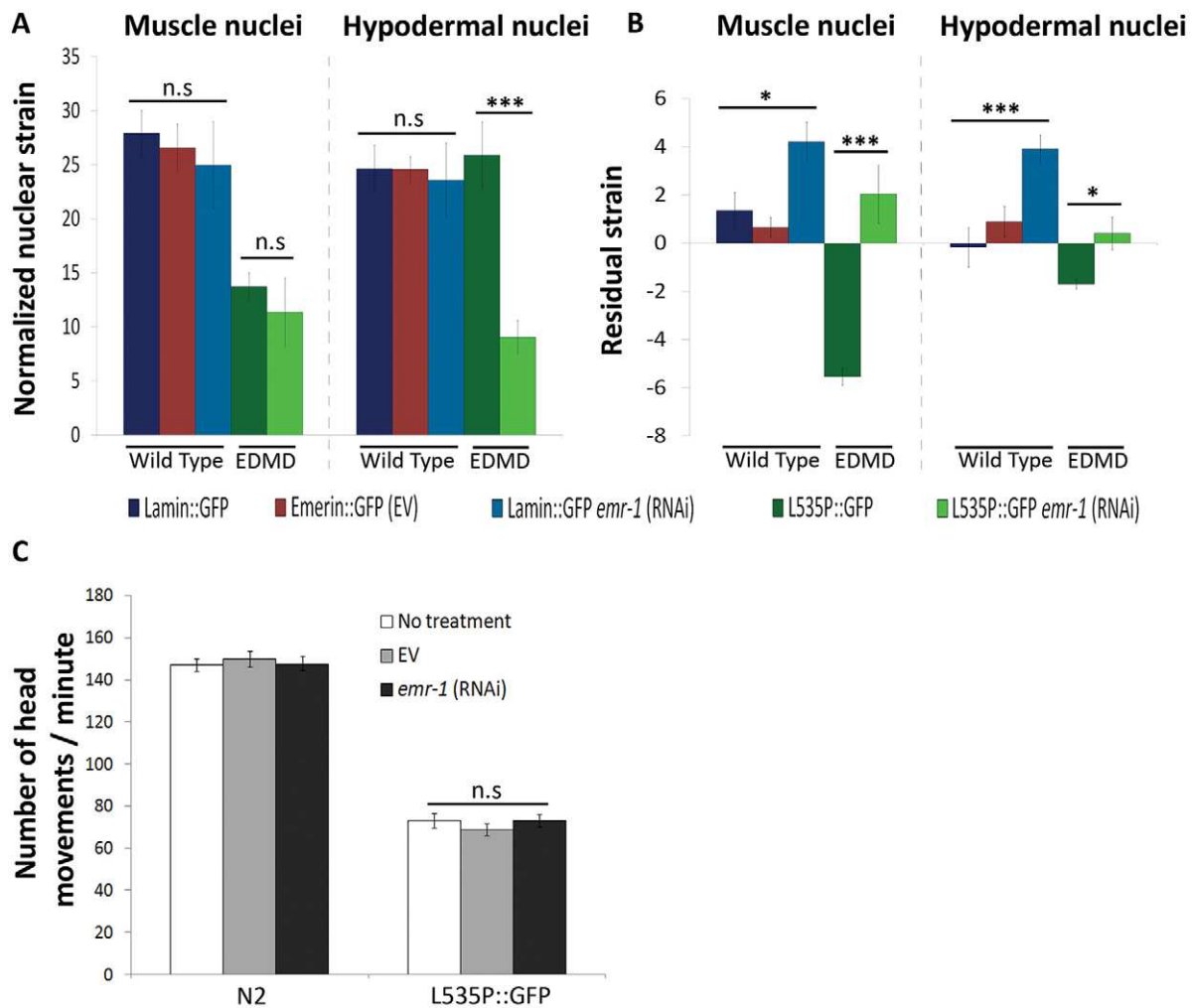


Fig. 5. *emr-1* (RNAi) affects the nuclear mechanical response but not the motility of L535P animals. (A) Normalized nuclear strain values were measured in response to *emr-1* (RNAi) treatment in muscle (left panel) and hypodermis (right panel) nuclei. Significant changes were only observed in L535P *emr-1* (RNAi) hypodermal nuclei ($P=6.6 \times 10^{-5}$; $n=24$ compared to untreated hypodermal L535P nuclei). (B) Recovery from mechanical strain was analyzed in muscle (left panel) and hypodermis (right panel) nuclei. Significant changes in both muscle and hypodermal nuclei were observed for both strains (in wild-type lamin $P=0.01$; $n=19$ and 0.0003 ; $n=20$ and in L535P animals $P=4.9 \times 10^{-6}$; $n=19$ and 0.006 ; $n=20$, respectively). (C) Analysis of animal motility in wild-type ($n=25$) and L535P ($n=30$) strains in response to *emr-1* (RNAi) treatment. P -values were calculated by performing a t -test. Results represent mean \pm s.e.m. * $P < 0.05$, *** $P < 0.0005$; n.s., not significant.

nuclei is higher and the lamin organization is somewhat different making these nuclei less or more resistant to strain; and (2) that the tissue-specific composition of the lamin-associated proteins (Korfali et al., 2012) might affect the ability of the nuclei to resist mechanical strain. These possibilities are not mutually exclusive.

Downregulation of the *fdps-1* gene leads to a reduction in the ability of muscle and hypodermis nuclei to resist mechanical strain (Table 2). Farnesylation of the lamin C-terminal CaaX motif is important for lamin association with the inner nuclear membrane (Firmbach-Kraft and Stick, 1993). Indeed, *fdps-1* (RNAi) caused a fraction of lamin to dissociate from the nuclear periphery in both wild-type and L535P animals. *fdps-1* (RNAi) did not affect the mechanical response of the progeria Q159K lamin mutation. Previous *in vitro* studies have revealed that the filament organization of Q159K is different from that of L535P or wild-type lamin. We speculate that, although *fdps-1* (RNAi) rescued the structure of L535P lamin filaments, it did not affect that of Q159K filaments and therefore did not affect the nuclear mechanical response. In line with the mechanical model, this change in lamin

localization can explain the rescue in mechanical response of the L535P muscle nuclei, which resulted in reversal of the severe muscle phenotypes and reduced animal motility (Table 2). Given that there are hundreds of proteins that undergo farnesylation including lamins A, B1 and B2 it will be interesting to determine the exact protein(s) where loss of their farnesylation leads to rescue of the L535P muscle phenotypes (Maltese, 1990). The farnesylated lamin A undergoes a cleavage of its last 15 residues, including the C-terminal farnesylated cysteine. However, loss of prenylation inhibits this cleavage and could change the mechanical properties of lamin filaments either directly or by altering their interactions with lamins B1 and B2 (Lattanzi, 2011). It would be important to subject L530P EDMD patient cells to prenylation inhibitors and analyze the potential rescue of their nuclear mechanical properties as a first step towards developing drug treatments for these patients.

Our results show that both the LINC complex and emerin are involved in determining the mechanical response of *C. elegans* nuclei (Table 2). These results show an evolutionary conserved role for these proteins in regulating the nuclear response to mechanical

Table 2. Summary of nuclear mechanical responses

RNAi	Normalized nuclear strain		Residual strain		Muscle structure	Motility
	Muscle nuclei	Hypodermal nuclei	Muscle nuclei	Hypodermal nuclei		
Wild-type animals						
No treatment	28%	25%	1.3%	−0.2%	Normal	Normal
<i>fdps-1</i>	Increased	Increased	Normal	Increased	Normal	Normal
<i>unc-84</i>	Normal	Slightly increased	Normal	Increased		Normal
<i>emr-1</i>	Normal	Normal	Slightly increased	Increased		Normal
EDMD-L535P animals						
No treatment	14%	26%	−5.5%	−1%	Aberrant structure, disorganized actin filaments, disorganized sarcomers	Reduced
<i>fdps-1</i>	Normal	Normal	Normal	Normal	Normal	Normal
<i>unc-84</i>	Slightly reduced	Normal	Increased	Normal		Further reduced
<i>emr-1</i>	No change	Reduced	Normal	Normal		Normal

strain (Folker et al., 2011; Ho et al., 2013; Lammerding et al., 2005; Zwerger et al., 2013). To our surprise, UNC-84 downregulation had no effect on nuclear response of wild-type muscle nuclei. It is possible that the disruption of the nucleo-skeletal coupling caused by *unc-84* (RNAi) was not sufficient to affect nuclear deformability in the presence of wild-type lamin. However, when lamin is mutated, as in the case of the EDMD L535P-linked lamin mutation, the muscle nuclear response is affected and nuclei appeared stiffer. This increased nuclear stiffness can be attributed to a certain degree of decoupling of the nucleus from the cytoskeleton. The reduced sensing of external strain in *unc-84* (RNAi), coupled with the potential decrease in lamin filament elasticity caused by the L535P mutation might cause the nucleus to remain contracted and appear more rigid even though the cytoskeleton is stretched. Given that the human mutation corresponding to L535P (L530P) shows a reduced association with SUN1 and SUN2 (Haque et al., 2010) it is not surprising that *unc-84* downregulation had only a small additive effect on the mechanical response of L535P muscle nuclei. This can also explain the improved recovery abilities of L535P muscle nuclei; despite the appearance of increased nuclear stiffness, these nuclei actually sense less strain owing to impaired nucleo-skeletal coupling, and so are able to better recover. In *C. elegans*, downregulation of emerin alone has no apparent effect on animal longevity and muscle function (Barkan et al., 2012). When subjecting worms to external strain application, *emr-1* (RNAi) affected nuclear recovery of both wild-type and L535P animals, however, it had no effect on muscle nuclear deformability. Previously, it has been shown that the human L530P lamin mutation partially reduces the abundance of emerin in the nuclear periphery of HeLa cells (Raharjo et al., 2001). It is possible that the L535P mutation has the same effect on emerin localization in muscle nuclei. Another possibility is that the effect of emerin on nuclear mechanical response is mediated through its interaction with one of its tissue-specific binding partners. The improved ability of L535P muscle nuclei downregulated for UNC-84 or emerin to return to their original shape following external strain application was not sufficient to ameliorate the defects in motility in these animals. However, the fact that, in L535P animals, the decreased muscle nuclear response following *unc-84* downregulation worsened motility whereas the increased muscle nuclear response following *fdps-1* downregulation improved motility, suggests a close correlation between nuclear stiffness, rather than residual

strain, and muscle tissue function. Both emerin and UNC-84 interact with lamin. Finding how the interaction of these proteins with other cell components affects nuclear mechanics, as well as their common mechanosensing and mechano-responding targets is a major goal for future studies.

MATERIALS AND METHODS

Strains

C. elegans strains (Table S1) were maintained and manipulated under standard conditions as previously described (Brenner, 1974). Mutant strains were out-crossed three times to ensure a clean background.

RNAi experiments

Experiments were performed by feeding as previously described (Timmons et al., 2001). The clones were obtained from the Vidal's and Ahringer's RNAi libraries (Kamath and Ahringer, 2003; Kim et al., 2005) and their efficiency was determined using real-time PCR (Fig. S3A). The empty vector L4440 was used as a control, and the DY3.2, R06C1.2, M01D7.6 and F54B11.3 vectors were used to downregulate *lmn-1*, *fdps-1*, *emr-1* and *unc-84*, respectively. For feeding experiments, we placed bleached embryos on feeding plates at 20°C, and late L4 progeny was collected after 48 h for analysis.

Swimming locomotion assay

Late L4 worms were gently placed in 150–200 µl of M9 buffer (a common buffer used in the *C. elegans* field: 3 g KH₂PO₄, 6 g Na₂HPO₄, 5 g NaCl, 1 ml of 1 M MgSO₄ in 1 litre of H₂O) on a glass slide at 22°C–23°C. Worm viability was verified by gentle touch with a pick. Twenty to thirty worms expressing the mutant lamin (L535P::GFP or Q159K::GFP) were scored. Controls included wild-type (N2) worms and worms fed with an empty vector. The number of head bends, in the opposite direction of the former movement was manually counted under a binocular microscope.

Transmission electron microscopy

Late L4 worms were collected from NGM plates and washed three times in M9 buffer. Worms were then pinched with #5 forceps behind the pharynx and at the tail and immediately fixed in 1 ml of 0.1 M sodium cacodylate buffer, 2.5% glutaraldehyde and 2.5% formaldehyde dissolved in ddH₂O and incubated overnight at 4°C. Worms were then washed three times in PBS, mounted in 3.4% low-melting-point agarose and fixed in 0.5 ml of 0.1 M sodium cacodylate buffer, 1% OsO₄ and 1.5% potassium hexacyanoferrate dissolved in ddH₂O for 1 h at 22°C. Samples were washed in 0.1 M sodium cacodylate and dehydrated by 10-min incubations in 30%, 50%, 70%, 80%, 90% and 95% analytical grade ethanol, followed by three 20-min washes in fresh 100% analytical grade ethanol and two 10-

min washes in propanyl oxide. Following dehydration, worms were incubated for 24 h in increasing concentrations of Agar-100 resin (25%, 50%, 75% and 100%) in propanyl oxide. Agar blocks were oriented appropriately before polymerization at 60°C for 48–72 h. The blocks were sectioned horizontally with a Diatome diamond knife to give fine 80–90-nm-thick slices of longitudinal view of each worm from head to tail. The sections were picked onto 200-mesh thin-bar copper grids and stained with uranyl acetate and lead citrate. Samples were viewed with an electron microscope (Philips Technai 12) equipped with a MegaView II CCD camera.

Phalloidin staining

Late L4 worms were collected and rinsed several times with M9 buffer. The worms were then freeze cracked using liquid nitrogen, thawed at room temperature and subsequently fixed for 5 min at –20°C in methanol, followed by 5 min acetone. After dehydration and blocking with blocking solution (PBS, 10% milk, 3% bovine serum albumin), the worms were incubated with fluorescent phalloidin (Cat. no. P1951, Sigma) diluted 1:200 in blocking solution for 1 h at room temperature. Actin filaments were imaged using an Axiocam CCD camera mounted on a Zeiss Axioplan II microscope equipped with epifluorescence.

Analysis of mechanical response

Late L4 worms were paralyzed using 2 mM levamisole. A single worm was then glued to a silicone membrane (Silicone sheeting 005" NRV G/G 40D 12"×12", Specialty Manufacturing, MI 48603-3440) by its head and tail using histoacryl tissue glue (REF 1050052, B. Braun Surgical, Spain). The membrane was mounted on the strain application device and the worm was imaged by an Orca R² high-resolution digital CCD camera mounted on an inverted Olympus IX81 fluorescent microscope using a 40×0.6 NA lens.

Controlled strain was applied on the membrane by using screws. The membrane was stretched to 140% and the worms were imaged again. Normalized nuclear strain was measured as follows. The change in nuclear length was measured as the length of the nucleus after stretching divided by the length of the nucleus before stretching minus 1. The length change of the area surrounding the nucleus was measured as the length between two neighboring nuclei along the stretch axis after stretching divided by the same length before stretching minus 1. The normalized nuclear strain value was obtained by dividing the change in nuclear length with that of the surrounding area and multiplying by 100 (Fig. 1A). Residual nuclear strain was calculated as the length of nuclei after relaxation from 1 min of stretching, divided by the length of the nuclei before stretching minus 1 and multiplied by 100 (Fig. 1B).

Analysis of nuclear lamin::GFP content

Late L4 worms expressing lamin::GFP or the L535P and Q159K lamin mutations tagged with GFP were paralyzed using 2 mM levamisole. The worms were subsequently imaged using an Axiocam CCD camera mounted on a Zeiss Axioplan II microscope equipped with epifluorescence. Using the ImageJ program, total nuclear gray values were measured as follows: mean nuclear gray value minus mean background signal multiplied by nuclear area.

Analysis of nuclear periphery enrichment of lamin::GFP

Late L4 worms expressing lamin::GFP or the L535P and Q159K lamin mutations tagged with GFP treated for either empty vector or *fdps-1* or *unc-84* RNAi were paralyzed using 2 mM levamisole. The worms were subsequently imaged using an Axiocam CCD camera mounted on a Zeiss Axioplan II microscope equipped with epifluorescence. Using the ImageJ program nuclear periphery to nucleoplasm lamin::GFP ratios were measured as follows: mean nuclear periphery gray value divided by mean nucleoplasmic gray value. Mean nuclear gray values were measured as the average nuclear gray value of the nucleus minus the mean background signal. The mean nucleoplasmic gray value was measured as the average nucleoplasmic gray value minus the mean background signal. The mean nuclear periphery gray value was measured as total nuclear gray value minus the total nucleoplasmic gray value divided by nuclear area minus the nucleoplasmic area. Nuclear periphery enrichment of lamin::GFP was measured as nuclear periphery to nucleoplasm lamin::GFP ratio minus 1.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

N.Z., M.Z., O.M. and Y.G. conceived and designed the experiments; N.Z., T.L. and M.Z. performed the experiments and analyzed the data; N.Z. and Y.G. wrote the paper.

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Supplementary information

Supplementary information available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.184309/-/DC1>

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